DECLE55.1CP2DV

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Moser, et al.	) Group Art Unit: 1644
Appl. No.	:	09/802,397	)
Filed	:	March 9, 2001	)
For	:	**DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE**	)
Examiner	:	Ewoldt, Gerald R.	

SECOND DECLARATION OF DR. MURIEL MOSER UNDER 37 C.F.R. § 1.132
(MOSER II)

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Muriel Moser, declare as follows

1. I am an inventor in the above-identified application.

2. The following experiments evidence that proliferating DCs provide better fusion partners for DC/tumor cell fusions and that the DC/tumor cell fusions formed are capable of providing immunostimulation. First, the phenotype of bone marrow progenitors at different times of culture was characterized (in presence of GM-CSF) and proliferation was monitored. The data is shown in Figures 1-3 attached. Figures 1-2 show evolution of several markers over the 9 day culture period: MHC-II, CD11c, F4/80, GR1 (Ly6G), CD90.2, CD4, CD8, and B220/CD45R. These results show that dendritic cells derived from bone marrow (BMDCs) are CD11c, MHC-II and F4/80 positive and 50% are GR1 positive. These BMDCs do not express B-cell or T-cell

markers (except CD90.2 at a negligible level). DC specific markers occur early in culture at day 2. 90% of cells are CD11c positive at day 4 and MHC-II positive at day 6.

3. In order to follow cell divisions, progenitor cells were labeled at D0 with CFSE, a cytoplasmic dye which divides equally into daughter cells and therefore decreases proportionally to cell division. The FACS analysis (Figure 3) shows the decrease of CFSE labeling over the course of culture. Cells were also stained for CD11c, MHC-II, F4/80, GR1, B220, CD4 and CD8 molecules. Cell divisions occurred mainly during the first 3 days of culture as shown by the left shift of CFSE staining. The rate of division seems to decrease significantly at day 4, which correlated with the expression of CD11c and F4/80 on almost all cells. At day 3, the high proportion of FL2 negative cells suggested that progenitor cells are dividing and remain poorly differentiated. Thus, cells at day 3 are proliferating. Conversely, at day 4, most cells are CD11c, F4/80, and to a lesser extent, MHC-II and GR1 positive.

4. Fusion experiments were performed using the BMDCs characterized above in paragraphs 2-3. Fusion of early BMDCs (days 3 and 4 of culture) was compared to fully differentiated BMDCs (day 9 of culture). HAT sensitive P815 tumor cells and BMDCs harvested at day 3, 4 or 9 of culture (end of culture) were mixed at 1:1 ratio (10^7 BMDCs and 10^7 P815), washed in serum free DMEM medium at 37°C and spun down. The cell pellet was broken by gentle agitation with a 2 ml serological pipet in presence of 500 µl of PEG 1500 (Boeringher Mannheim). 500 µl of DMEM medium (37°C) were added 1.5 min later. Increasing volumes of DMEM (1, 2, 4 ml) were added every 1.5 min. Cells were then spun down, diluted in PBS BSA (1%)-EDTA (10 mM) and sorted by magnetic microbeads linked to anti-CD11c antibodies, diluted in complete HAT medium and plated. After 24 hours of culture, hybrids were cloned by limiting dilutions in 96 well plates at 0,3 and 1 cell per well in complete HAT medium.

5. ***Fusion yield***

The table shows that higher numbers of hybrid cells were obtained after fusion between P815 and BMDCs at day 3 of culture than with BMDCs at days 4 or 9 of culture, confirming that proliferating DCs are more efficient in DC/tumor cell fusions.

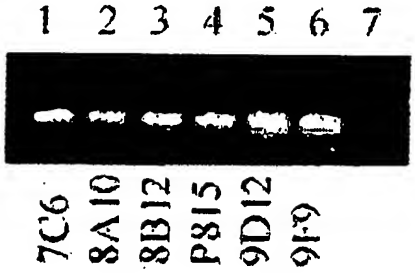
MM

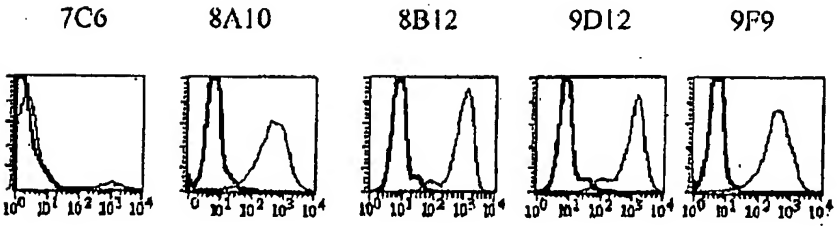
	Number of clones obtained with BMDCs at day 3 of culture in 3X96 well plates (1 cell/well)	Number of clones obtained with BMDCs at day 4 of culture in 3X96 well plates (1 cell/well)	Number of clones obtained with BMDCs at day 9 of culture in 3X96 well plates (1 cell/well)
Exp 1	54	12	-
Exp 2	28	5	1
Exp 3	135	5	-

6. ***Phenotype analysis of hybrid cells***

The fused cells were analyzed to determine that they were true hybrids between DCs (defined as CD11c positive) and P815 tumor cells. CD11c expression was analyzed by flow cytometry and expression of mRNA specific for P815-associated antigen P1A was assessed by RT-PCR. Among 27 clones (exp 3), 26 were CD11c+ and 19 were P1A+.

Example of phenotype analysis for 5 clones





mRNA expression of P1A is shown for the five clones (lanes 1, 2, 3, 5, 6), P815 tumor cells (lane 4) and 3B4 irrelevant cell, the negative control (lane 7). All clones express tumor antigen P1A. The FACS analysis shows that four clones express CD11c.

7. We conclude that fusion of P815 tumor cells and BMDCs cultured for only 3 days, while the DCs are proliferating, produces more hybrid cells than fusion with BMDCs cultured from 4 or 9 days. Thus, we conclude that it is preferable to use proliferating dendritic cells to make DC/tumor cell fusions because the yield of fused cells is much higher when proliferating DCs are used.

8. Allo-MLRs were performed to assess the immunostimulatory properties of hybrid cells. Hybrid cells (I-Ad) treated or not with LPS (50 μg/ml overnight) were cultured with purified naive T cells (I-Ab). Results from testing of 19 P1A+/CD11c+ hybrid clones:

2 exhibit poor immunostimulatory properties

4 exhibit weak immunostimulatory properties

13 exhibit strong immunostimulatory properties

We conclude that DC/tumor cell fusions may be produced efficiently using proliferating DCs isolated from bone marrow and that these fused cells have strong immunostimulatory properties.

9. We also conducted experiments using different sources for isolation of DCs. While paragraphs 1-8 above describe procedures using DCs from bone marrow, sources such as blood, lymph, lymph nodes and spleen may also be used. We have found that bone marrow, blood and lymph contain a low number of differentiated DCs, but a high number of DC progenitors. Spleen and lymph nodes contain a high number of differentiated DCs and a low number of DC progenitors. As demonstrated in the instant specification, DCs isolated from spleen produced a T-cell/tumor cell hybridoma, not a DC/tumor cell hybrid (see Examples 1-6 and paragraph 0184 of the published application). Further experiments in my laboratory have confirmed that spleen is a poor source for DCs to produce DC/tumor cell hybrids while bone marrow, blood and lymph are the preferred sources.

10. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States codes and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: February 4, 2005 By: Muriel Moser

Muriel Moser

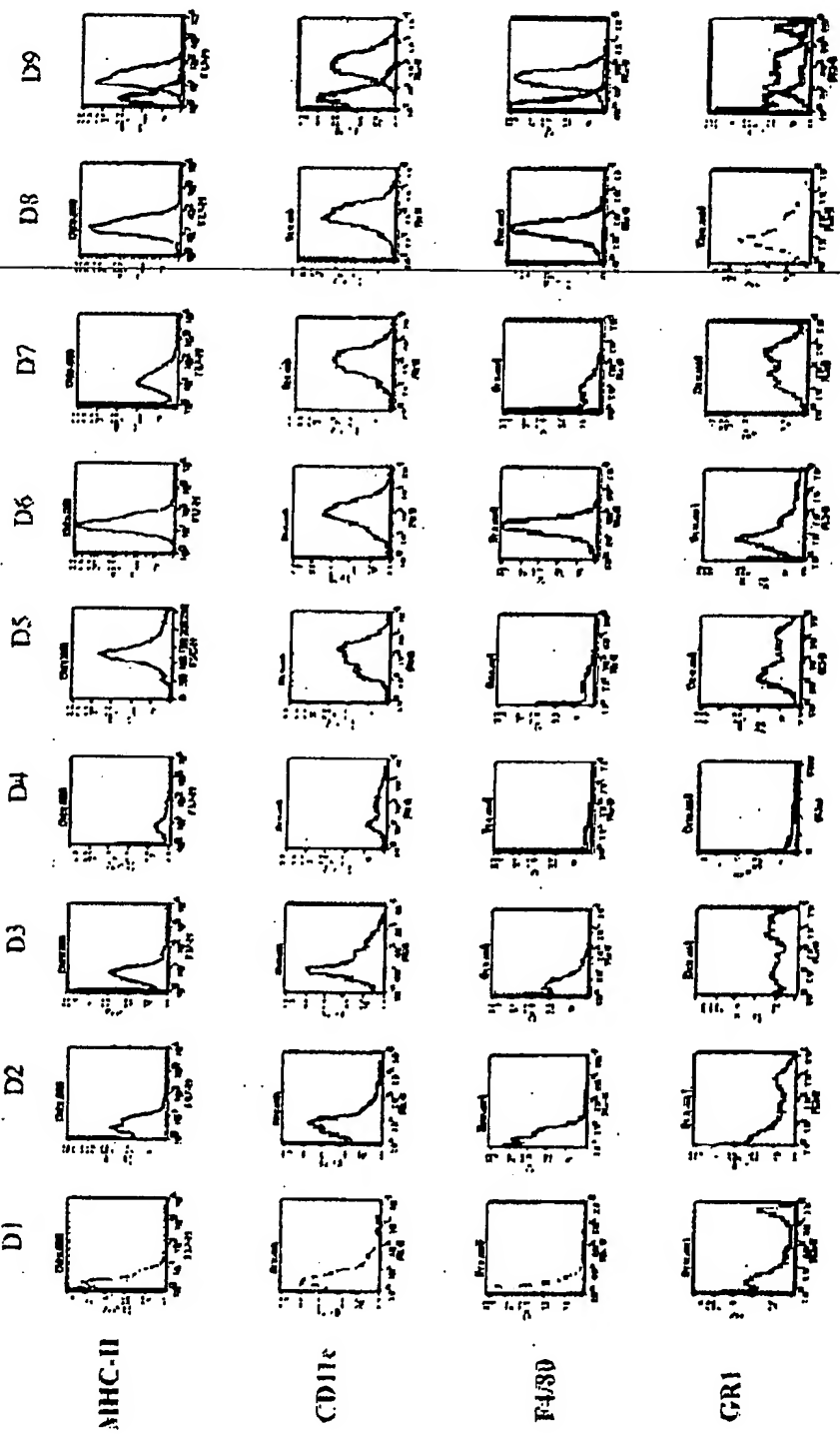


FIGURE 1

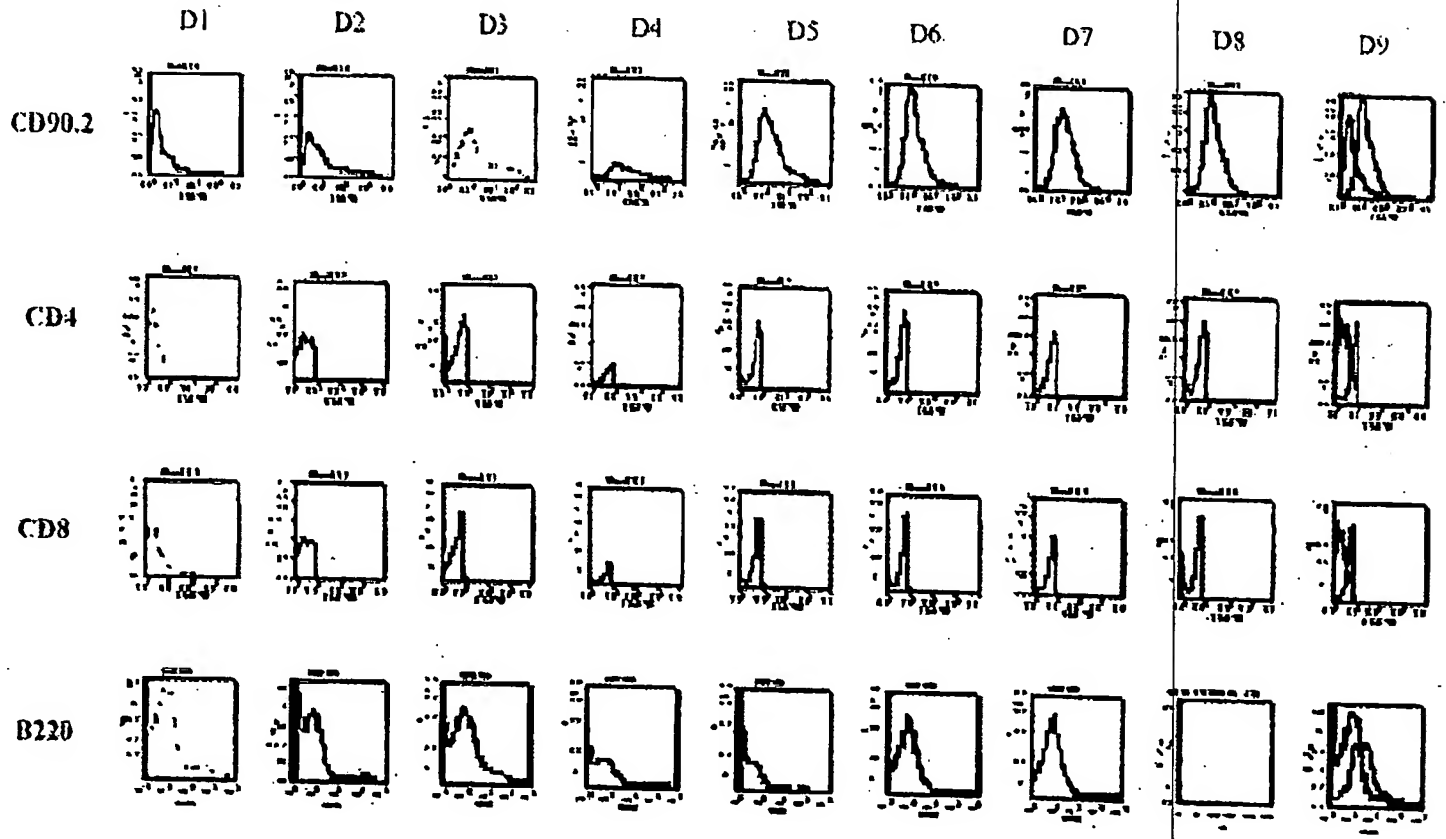


FIGURE 2

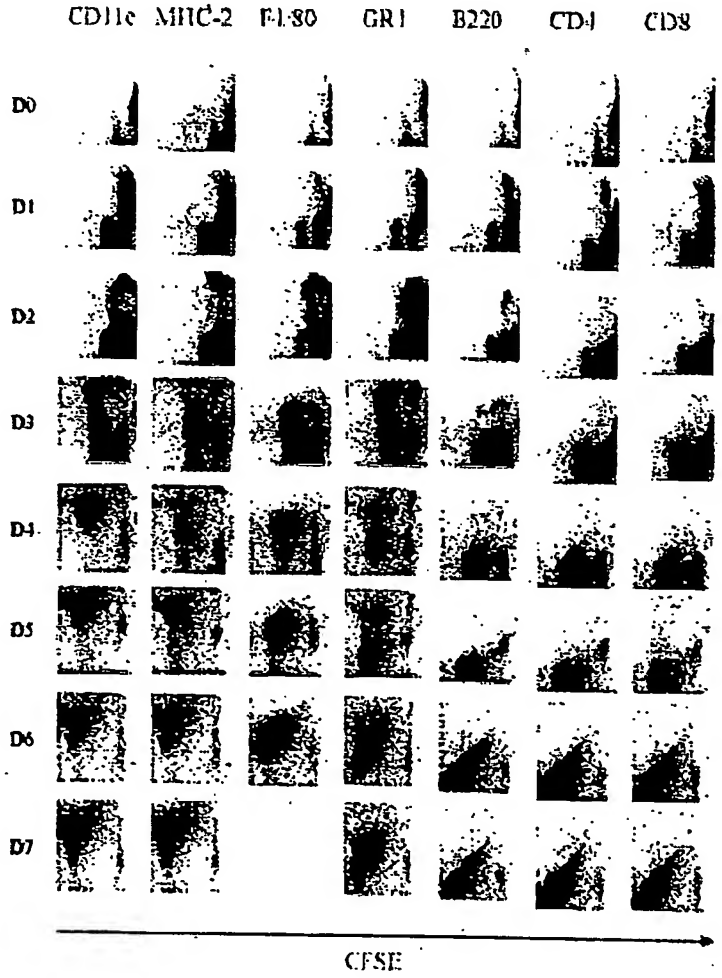


FIGURE 3

CURRICULUM VITAE

Name:	Muriel Moser
Date and place of birth:	15/4/54 Brussels, Belgium
Citizenship:	Belgian
Marital status:	married
	3 children
Education:	1977 B.S. (zoology) Université Libre de Bruxelles La plus grande distinction
	1983 Ph.D (zoology) Université Libre de Bruxelles La plus grande distinction

Brief chronology of employment

1977-1980	Predoctoral IRSIA fellow
1980-1982	Aspirant F.N.R.S.
1982-1983	Researcher, Laboratoire de Physiologie Animale Université Libre de Bruxelles
1984-1986	Visiting Fellow, Immunology Branch, National Cancer Institute, NIH Bethesda, Maryland, U.S.A.
1986-1991	Researcher, laboratoire de Physiologie Animale Université Libre de Bruxelles
1991-2002	Research Associate F.N.R.S.
2002-	Senior Research Associate Belgian National Fund for Research (F.N.R.S.)

Dr Muriel Moser, Maître de recherches du FNRS
Séjours d'études à l'étranger
1996-2000

4th International Symposium on dendritic cells in Fundamental and Clinical Immunology
Member of the International Scientific Committee
October 5-10, 1996
Venice, Italie

Keystone Symposia
Cellular and Molecular Biology of dendritic cells
Santa Fe, New Mexico (Short oral presentation)
March 7-13 1998

5th International Symposium on Dendritic cells in Fundamental and Clinical Immunology
Pittsburgh, Pennsylvania, USA
September 24-28, 1998

4th European Winter Conference in Immunology (invited speaker)
Basis of immune memory: bacterial and tumoral vaccines developments
Saint Sorlin, France
January 19-22, 1999

Keystone Symposia (invited speaker)
T lymphocyte activation, differentiation and death
Keystone, Colorado
January 28, February 3, 2000

Seminar (invited speaker)
Institut de Biochimie, Epalinges (CH)
April 27-28, 2000

ENII Conference 2000 (invited speaker)
Ile des Embiez
17-21 mai 2000

9th International Symposium on
Molecular Cell Biology of Macrophages (invited speaker)
Kyoto, Japon
June 5 and 6, 2000

6th International Symposium on Dendritic cells (invited speaker)
Port Douglas, Queensland, Australia
May 26-June 1, 2000

Participation à des jurys de thèse de doctorat en France

EUNIDI (réseau supporté par la Commission Européenne)

Final network Meeting
Leiden, The Netherlands
October 16-17, 2000

Comité d'Evaluation Scientifique des laboratoires
du Dr Jacques Mallet et du Dr David Klatzmann (Paris)
Le 27 juillet 2000
Mission confiée par le CNRS

University of Tennessee, Knoxville
Séminaire et collaboration (invited)
14-19 octobre 2000

Institut Pasteur, Paris, France
Jury de thèse et séminaire (invitée)
23 et 24 octobre 2000

Karolinska Institute, Stockholm, Suède
Cours d'immunologie et séminaire (invitée)
11-12 décembre 2000

Hôpital Cochin, Paris, France
Séminaire (invitée)
21 décembre 2000

Erasmus University, Rotterdam, The Netherlands
Minisymposium (invited speaker)
20 février 2001

Keystone Symposium (invited speaker)
Dendritic cell : Interfaces with Immunobiology and medicine
Taos, New Mexico, March 12-18, 2001

University of Zurich (Prof. Zinkernagel)
Seminar "Dendritic cell subsets and T helper development in vivo"
Zurich, Switzerland, May 7-8 2001

3rd Berlin Symposium on Immunology of Infection
Dendritic cells and macrophages in infectious diseases
Berlin, May 25th to 27th, 2001; invited speaker

Université de Munich
Seminar
Munich, Germany, June 18, 2001

Institut Pasteur de Lille
Thèse de doctorat H. Hammad, rapporteur
29 juin 2001

11th International Congress of Immunology

Chairman
Stockholm, Sweden; July 22-27, 2001

Manchester, United Kingdom
The dendritic cell-star player of immunity (invited speaker)
September 10-11, 2001

University of Lund, Sweden
Opponent for PhD thesis and seminar
September 20-22, 2001

32nd Annual meeting of the German Society of Immunology
Invited speaker
Dresden, Germany, September 26-29, 2001

Euresco conference : communication within the immune system : basic rules and their breakdown (Invited speaker)
San Feliu, Espagne Octobre 13-18, 2001

Rapporteur de thèse, Nadège Noirey
Unité 346, Inserm, Lyon
Le 22 octobre 2001

Rapporteur de thèse, Nathalie Bendriss
Schering-Plough
Le 26 octobre 2001

University of Iowa
Course and seminar (invited)
October 27-31, 2001

Karolinska Institute
Course and seminar (invited)
December 4, 2001

University of Oxford
Examiner of Victoria Strong's thesis
December 7, 2001

Congrès du Club Francophone des cellules dendritiques
(Présidente)
Paris, 10-11 décembre 2001

Centre d'Immunologie de Lumigny
Marseille, France, 30-31 janvier 2002
Jury de thèse de doctorat

Keystone Symposia (invited speaker)
Rethinking the Pathogenesis of Asthma
Santa Fe (Colorado), February 8-13, 2002

Expert Meeting on dendritic cell vaccines in renal
cell carcinoma and other tumors
Innsbruck, 26-27 April, 2002 (invited speaker)

Dendritic cells at the host pathogen interface
Airlie, Virginia, May 4-7, 2002 (invited speaker)

Immunohistologie des cellules dendritiques humaines
Atelier organisé par le Club Francophone des cellules dendritiques
Lyon, France, le 28 mai 2002

Faces and phases of dendritic cells
Amsterdam, May 29, 2002
Invited speaker

Dendritic cells in Control of Human Disease
Worhshop organized by The European Commission
Brussels, 30-31 May, 2002 (invited speaker)

Euroconference
Interactions between innate and adaptive immunity
in mammalian defense against bacterial infections
Berlin, June 5-8, 2002 (invited speaker)

Mission Inserm
Création unité 2003
Lyon, le 10 juin 2002

Institut Pasteur, Paris
Séminaire
« Regulation of T helper development by dendritic cells in vivo »
le 28 juin 2002

5th EFIS Tatra Immunology Conference
Molecular determinants of T cell Immunity
September 2-7, 2002, Tatra Mountains
Slovakia Invited Speaker

Mission Inserm, Expert européen
Création d'Unité, Institut Pasteur, Paris
Le 17 septembre 2002

7th International Symposium on
Dendritic Cells, September 19-24, 2002
Bamberg, Germany
Invited speaker and Chaiperson

Joint meeting ICS, ISICR, ECS, SLB
Cytokines and Interferons

October 6-10, 2002, Invited speaker

Réunion Interface INSERM/SPLF
La cellule dendritique, conférencier invité
Paris, 16-17 octobre 2002

Genzyme, Boston, USA
October 28-29 2002

Quatorzième cours annuel de la SFI
Centre de Congrès des Pensières, Annecy, France
08-12 novembre 2002
Conférencier invité

British Society for Immunology
Congress 2002
3-6 December
Harrogate , Yorkshire

Contrat Européen

European Union Network for Investigation of Dendritic cell Immunotherapy (EUNIDI) for Induction of Anti-viral and Anti-tumor Immunity and Transplantation Tolerance

Contract number: FERB FMRX CT 960053

Contract duration: 48 months from 01.10.1996 to 30.09.2000

Network Coordinator: JM Austyn (Oxford)
Participating teams: J. Austyn, University of Oxford (UK)
J. Shields, Cantab Pharmaceutical Res. Ltd, Cambridge (UK)
C. Figdor, University of Nijmegen (NL)
A. Lanzavecchia, Basel Institute for Immunology (CH)
M. Moser, University of Brussels (BE)
P. Ricciardi-Castagnoli, Consorzio Milano Ricerche, IT
G. Schuler, University of Erlangen (DE)
M. Suter, University of Zurich (CH)
P. Mavromara, Hellenic Pasteur Institute (GR)
C. Melief, University of Leiden (NL)

Muriel Moser
Chercheur Qualifié F.N.R.S.

I. INTERNATIONAL PUBLICATIONS (PEERED REVIEWED)

1983

Idiotypic manipulations in mice: Balb/c mice can express the crossreactive idiotype of A/J mice.
M. Moser, O. Leo, J. Hiernaux and J. Urbain.
Proc. Natl. Acad. Sci. USA. 80 (1983), 4474-4478.

1984

Idiotypic manipulations in the arsonate system.
O. Leo, J. Urbain, M. Moser, J. Marvel, J. Hiernaux and M. Slaoui.
Ann. Immun. (Inst. Pasteur). 135C (1984), 45-50.

Idiotopic analysis of potential and available antiarsonate repertoires.
M. Slaoui, O. Leo, J. Marvel, M. Moser, J. Hiernaux and J. Urbain.
J. Exp. Med. 160 (1984), 1-11.

1985

Idiotypic analysis of polyclonal and monoclonal anti-p-azophenylarsonate antibodies of Balb/c mice expressing the major cross-reactive idiotype of A/J strain.
O. Leo, M. Slaoui, J. Marvel, E.C. Milner, L. Hiernaux, M. Moser, J.D. Capra and J. Urbain.
J. Immmunol. 134 (1985), 1734-1739.

Cellular interactions in Graft-versus-Host-Induced T cell immune deficiency.
M. Moser, T. Iwasaki, and G.M. Shearer.
Immunol. Rev. 88 (1985), 134-151.

1986

AIDS as a consequence of Ia antigen recognition : a closer look.
G. M. Shearer and M. Moser.
Immunol. Today 7 (1986), 34-36.

Idiotypic games within the immune network.
M. Slaoui, G. Urbain-Vansanten, C. Demeur, O. Leo, J. Marvel, M. Moser, J. Tassignon, M.I. Greene, J.D. Capra, and J. Urbain.
Immunological Reviews. 90 (1986), 73-91.

Study of idiotypic suppression induced by anti-cross reactive idiotype monoclonal antibody in the anti-p-azophenylarsonate antibody response.
J. Hiernaux, J. Marvel, M. Moser, O. Leo, M. Slaoui and J. Urbain.
J. Immunol. 136 (1986), 1960-1967.

Molecular mapping of idiotypes of anti-arsonate antibodies.
D. Jeske, E.C. Milner, O. Leo, M. Moser, J. Marvel, J. Urbain and J.D. Capra.
J. Immunol. 136 (1986), 2568-2574.

1987

The influence of V_k gene polymorphism on the induction of silent idiotypes in the arsonate system.
J. Marvel, J. Tassignon, M. Brait, K. Meek, E.C.B. Milner, M. Moser, J.D. Capra, and J. Urbain.
Molec. Immunol. 24 (1987), 463-469.

Graft-versus-host reaction limited to a class II MHC difference results in a selective deficiency in $L3T4^+$ but not in $Lyt2^+$ T helper cell function.
M. Moser, T. Mizuochi, S.O. Sharrow, A. Singer, and G.M. Shearer.
J. Immunol. 138 (1987), 1355-1362.

1988

Role of L3T4+ and $Lyt2^+$ donor cells in graft-versus-host immune deficiency induced across a class I, class II or whole H-2 difference.
M. Moser, S.O. Sharrow, and G.M. Shearer.
J Immunol. 140 (1988), 2600-2608.

Some aspects of idiotypic networks: self/non-self discrimination, selection of available repertoires and broken mirrors.
J. Urbain, F. Andris, M. Brait, C. Demeur, D. De Wit, O. Leo, J. Marvel, F. Mertens, M. Moser, M. Slaoui, J. Tassignon, G. Urbain-Vansanten, M. Wikler, F. Willems and C. Wuilmart.
Ann. Inst. Pasteur Immunol. 139 (1988), 609-618.

1990

Immune surveillance: both $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells control in vivo growth of P815 mastocytoma.
V. Flamand, C. Biernaux, M. Van Mechelen, T. Sornasse, J. Urbain, O. Leo, and M. Moser.
Int. J. Cancer 45 (1990), 757-762.

Anti-CD3 antibodies induce T cells to secrete interleukin 4 both in vitro and in vivo. V. Flamand, D. Abramowicz, M. Goldman, C. Biernaux, G. Huez, J. Urbain, M. Moser and O. Leo.
J.Immunol. 144 (1990), 2875-2882.

Hypothermia and hypoglycemia induced by anti-CD3 monoclonal antibody in mice: role of tumor necrosis factor.
M. Alegre, P. Vandenabeele, V. Flamand, M. Moser, O. Leo, D. Abramowicz, J. Urbain, W. Fiers and M. Goldman.
Eur. J. Immunol. 20 (1990), 707-710.

1991

Cytokine release syndrome induced by the 145-2C11 anti-CD3 monoclonal antibody in mice: prevention by high doses of methylprednisolone.
M. Alegre, P. Vandenabeele, M. Depierreux, S. Florquin, M. Deschodt-Lanckman, V. Flamand, M. Moser, O. Leo, J. Urbain, W. Fiers and M. Goldman.
J. Immunol., 146 (1991), 1184-1191.

Hyper IgE in stimulatory graft-versus-host disease : role of interleukin-4.
J.-M. Doutrelepont, M. Moser, O. Leo, D. Abramowicz, M.L. Vanderhaegen, J. Urbain and M. Goldman.
Clin. Exp. Imm., 83 (1991), 133-136.

Modulation of murine host-versus-graft disease by anti-CD3 monoclonal antibody. M. Wissing, A. Marchant, M. Moser, V. Flamand, O. Leo, D. Abramowicz, J. Urbain and M. Goldman.
Clin. Exp. Imm. 83 (1991), 333-337.

Evidence that pentoxifylline reduces anti-CD3 monoclonal antibodies-induced cytokine release syndrome.
M. Alegre, K. Gastaldello, D. Abramowicz, P. Kinnaert, P. Vereestraten, L. De Pauw, P. Vandenabeele, M. Moser, O. Leo and M. Goldman.
Transplantation 52 (1991), 674-679.

Treatment of mice bearing BCL1 lymphoma with bispecific antibodies.
J. Brissinck, C. Demanet, M. Moser, O. Leo, K. Thielemans.
J. Immunol., 147 (1991), 4019-4026.

1992

Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo.
T. Somasse, V. Flamand, G. De Becker, H. Bazin, F. Tielemans, K. Thielemans, J. Urbain, O.Leo, and M. Moser.
J. Exp. Med, 175 (1992), 15-21.

1993

Preferential activation of TH2 cells in chronic Graft-versus-host reaction.
D. De Wit, M. Van Mechelen, C. Zanin, J.M. Doutrelepont, T. Velu, C. Gérard, D. Abramowicz, J.-P. Scheerlinck, P. De Baetselier, J.Urbain, O. Leo, M. Goldman, and M. Moser.
J. Immunol. 150 (1993), 361-366.

In vivo induction of IL-10 by anti-CD3 monoclonal antibody in mice.
T. Velu, P. Durez, M. Van Mechelen, C. Gérard, D. Abramowicz, M. Moser, O. Leo and M. Goldman.
Transpl. Proc. 25 (1993), 568-569.

1994

Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo.
V. Flamand, T. Sornasse, K. Thielemans, C. Demanet, M. Bakkus, H. Bazin, F. Tielemans, O. Leo, J. Urbain, and M. Moser.
Eur. J. Immunol. 24 (1994), 605-610.

Immunoglobulin isotype regulation by antigen-presenting cells in vivo.
G. De Becker, T. Sornasse, N. Nabavi, H. Bazin, F. Tielemans, J. Urbain, O. Leo and M. Moser.
Eur. J. Immunol. 24 (1994), 1523-1528.

Role of T cell subsets in the bispecific antibody (anti-Id x anti-CD3) treatment of the BCL1 lymphoma.
C. Demanet, J. Brissinck, O. Leo, M. Moser and K. Thielemans.
Int. J. Cancer 54 (1994), 2973-2978.

In vivo immunosuppression induced by a non mitogenic antibody to mouse CD3: evidence that induction of long-lasting in vivo unresponsiveness requires TcR signaling. V. Flamand, V. Donckier, D. Abramowicz, M. Goldman, P. Vandenabeele, J. Urbain, M. Moser and O. Leo.
Cell. Immunol. 157 (1994), 239-248.

Role of T cell subsets in the bispecific antibody (anti-Id x anti-CD3) treatment of the BCL1 lymphoma.
Demanet, C., J. Brissinck, O. Leo, M. Moser and K. Thielemans.
Cancer Res. 54 (1994), 2973-8.

1995

Costimulation lowers the threshold for activation of naive T cells by bacterial superantigens.
E. Muraille, G. De Becker, K. Thielemans, M. De Boer, J. Urbain, M. Moser and O. Leo.
Int. Immunol. 7 (1995), 295-304.

Activation of murine T cells by bacterial superantigens requires B7-mediated costimulation.
E. Muraille, T. De Smedt, K. Thielemans, J. Urbain, M. Moser and O. Leo.
Cellular Immunology, 162 (1995), 315-320.

The B7.2 molecule provides costimulatory functions in vivo in response to the bacterial exotoxin SEB.
E. Muraille, T. De Smedt, J. Urbain, M. Moser and O. Leo.
Eur. J. Immunol. 25 (1995), 2111-2114.

Production and characterization of bispecific single-chain antibody fragments.
De Jonge, J., J. Brissinck, C. Heirman, C. Demanet, O. Leo, M. Moser, and K. Thielemans.
Mol. Immunol. 32 (1995), 1405-1412

Glucocorticoids downregulate dendritic cell function in vitro and in vivo.
M. Moser, T. De Smedt, T. Sornasse, F. Tielemans, A.A. Chentoufi, E. Muraille, M. Van Mechelen, J. Urbain and O. Leo.
Eur. J. Immunol. 25 (1995), 2818-2824.

1996

Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo.
T. De Smedt, B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo and M. Moser.
J. Exp. Med. 184 (1996), 1413-1424.

Costimulation regulates the kinetics of IL-2 response to bacterial superantigens.
E. Muraille, S. Devos, K. Thielemans, J. Urbain, M. Moser and O. Leo.
Immunology 89 (1996), 245-249

1997

Staphylococcal Enterotoxin B induces an early and transient state of immunosuppression characterized by Vb-unrestricted T cell unresponsiveness and defective antigen-presenting cell functions.
E. Muraille, T. De Smedt, F. Andris, B. Pajak, M. Armant, J. Urbain, M. Moser and O. Leo
J. Immunol. 185 (1997), 2638-2647

Purification and characterization of bovine dendritic cells from peripheral blood
X. Renjifo, C. Howard, P. Kerkhofs, M. Denis, J. Urbain, M. Moser and P.P. Pastoret
Veterinary Immunology and Immunopathology 60 (1997), 77-88.

Effect of interleukin-10 on dendritic cell maturation and function
T. De Smedt, M. Van Mechelen, G. De Becker, J. Urbain, O. Leo and M. Moser.
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REMETHYLATION AT SITES 5′ OF THE MURINE Lyt-2 GENE IN ASSOCIATION WITH SHUTDOWN OF Lyt-2 EXPRESSION[1]

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We have used hybridomas made by fusing the Lyt-2⁻ AKR thymoma, BW5147, to Lyt-2⁺ SJL/J lymph node cells to study the regulation of Lyt-2 expression. Fusions of this type yielded hybridomas, the majority of which failed to express Lyt-2. In the minority of hybridomas that did express surface Lyt-2, expression was transient and greatly diminished in terms of molecules of Lyt-2 per cell. Lack of Lyt-2 expression was not due to loss of the gene encoding this cell surface molecule; rather, negative regulation of Lyt-2 appeared to be at the level of transcription (i.e., no Lyt-2 transcripts were detected in these hybridomas). We have shown that the Lyt-2 gene is undermethylated in normal Lyt-2⁺ T cells, whereas the gene is heavily methylated in Lyt-2⁻ liver cells and in BW5147. Loss of Lyt-2 expression in (BW5147 × Lyt-2⁺ SJL/J lymph node cell) hybridomas was associated with remethylation of DNA within the Lyt-2 gene and at sites 5′ of the Lyt-2 gene.

Lyt-2 and L3T4 are two T cell-specific surface molecules expressed on mature T cells in a mutually exclusive manner. Lyt-2 is generally found on T cells restricted to recognition of Ag in the context of class I MHC molecules, whereas L3T4 is found on class II MHC-restricted T cells (1). T cells acquire the expression of Lyt-2 and L3T4 during passage through the thymus (2–4), where MHC restriction and tolerance to self-Ag is "learned" (5, 6). The adult murine thymus is comprised of 2 to 3% T cells that express neither Lyt-2 nor L3T4 ("double-negative" phenotype), approximatley 80% T cells that express both Lyt-2 and L3T4 ("double-positive" phenotype), and about 15% T cells of the "mature" phenotype, i.e., those that express either Lyt-2 or L3T4 but not both (2–4). During ontogeny, the first cells to appear in the thymus, in terms of Lyt-2 and L3T4 expression, are those of the double-negative phenotype; the influx of these cells is followed by a gradual increase in the double-positive phenotype (starting at day 15 to 16 of gestation); finally, at day 18 to 19, mature T cells are detected in the thymus (7). It has been shown that the double-negative thymic T cells can give rise to both the double-positive cells and to cells of the mature phenotype (8). However, it is not yet clear whether the differentiation of these precursor T cells involves passage through an intermediate stage where both Lyt-2 and L3T4 are expressed, or whether mature T cells arise directly from the double-negative precursor, in which case the double-positive phenotype must then represent those cells that have failed the complex program of maturation in the thymus. The TCR is first detected in the fetal thymus on day 15 of gestation and is expressed on some double-positive T cells and on cells of the mature phenotype in the adult thymus (9, 10). It is not yet understood how Lyt-2 and L3T4 expression is controlled during thymic development, and how expression of one or the other of these molecules is coordinated with the expression of the appropriate class I or class II MHC-restricted Ag receptor.

In the present study we have used a model hybridoma system to study whether expression of the Lyt-2 gene is under positive or negative regulatory control. Fusion of murine Lyt-2⁺ T cells to the AKR thymoma, BW5147, yielded hybrids that failed to express Lyt-2, suggesting negative control by means of a *trans*-acting regulatory factor. We have identified sites 5′ of the Lyt-2 gene that are methylated in Lyt-2⁻ cells but undermethylated in cells that express the Lyt-2 molecule. Two of these sites are remethylated in association with shutdown of the Lyt-2 gene in T cell hybridomas.

MATERIALS AND METHODS

Mice. All mice were either obtained from The Jackson Laboratory, Bar Harbor, ME, or bred in our facility at National Jewish Center.

T cell hybridoma production and analysis. Lyt-2⁺ T cells from SJL/J lymph node were prepared in one of two ways. In early fusions LNC[3] were cultured with Con A for 2 days and Lyt-2⁺ cells were expanded in medium supplemented with IL-2 for an additional 5 days. In later fusions, Lyt-2⁺ cells were isolated from LNC by panning on GK1.5 (anti-L3T4)-coated dishes. Nonadherent cells were cultured for 2 days with Con A and irradiated syngeneic filler cells, and, if necessary, blasts were expanded for an additional 2 days in the presence of IL-2. The resulting populations of LNC, which ranged from 89 to 98% Lyt-2⁺ T cells, were fused to the AKR thymoma, BW5147, as described previously (11). Hybridomas were screened by ELISA or by flow cytometry by using the following mAb: HO22.1 (mouse IgM specific for Thy-1.1) (12), HO13-4-6 (mouse IgM specific for Thy-1.2) (12), 116-13.1 (mouse IgG specific for Lyt-2.1) (gift from Dr. F. W. Shen), 2.43 (rat IgG specific for Lyt-2.2) (13), and GK1.5 (rat IgG specific for murine L3T4) (14). Secondary antibodies were alkaline-phosphatase-linked goat anti-rat or anti-mouse IgG (Sigma) (for ELISA), fluorescein-conjugated monoclonal mouse anti-rat κ chain (RG7/9.1) (gift from Dr. T. Springer) and monoclonal rat anti-mouse κ chain (187.1) (15) (for cytofluorimetry). Any hybrid that

[3] Abbreviations used in this paper: LNC, lymph node cells.

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Phenotype	Number of Hybrids (30 / 60 / 90)	Positive / Total (%)
Thy1.1⁺(B) Thy1.2⁺(S)		89/89 (100)
Lyt 2.1⁺ (B)		0/89
Lyt 2.1⁺ (B) L3T4⁺		0/89
Lyt 2.2⁺(S)		0/89
Lyt2.2⁺(S) L3T4⁺		14/89 (16)
Lyt2⁻ L3T4⁻		65/89 (73)
L3T4⁺		10/89 (11)

Figure 1. Phenotypes of (BW5147 × Lyt-2⁺ SJL LNC) hybridomas. This is a compilation of data from three separate fusions in which SJL LNC ranged from 89 to 98% Lyt-2⁺ and 2 to 8% L3T4⁺. (*B*) BW5147 allele, (*S*) SJL allele.

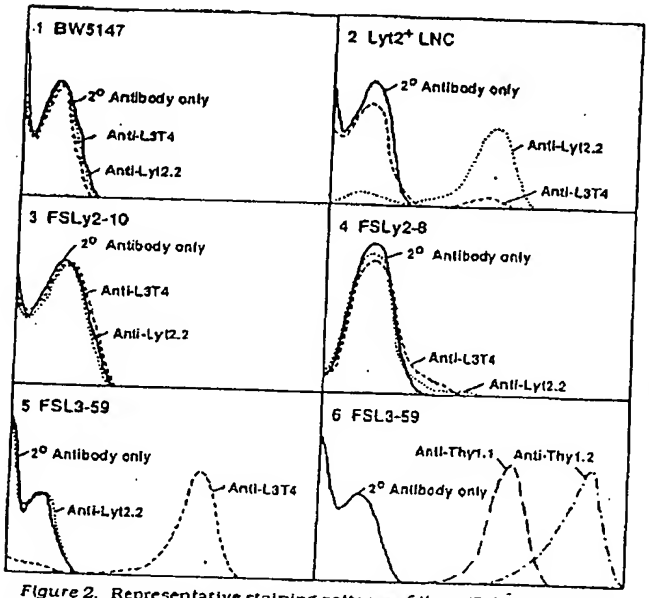


Figure 2. Representative staining patterns of three (BW5147 × Lyt-2⁺ LNC) hybridomas. The hybridomas (FSLy2-10, FSLy2-8, FSL3-59) or parental cells (BW5147 and Lyt-2⁺ LNC) were incubated with anti-L3T4 (----), anti-Lyt-2.2 (·····), anti-Thy-1.1 (-·-·-·-), anti-Thy-1.2 (-··-··-), or no primary antibody (——), followed by fluorescein-conjugated anti-rat κ chain or fluorescein-conjugated anti-mouse κ chain.

stained 2% or more over background was scored positive. More than 98% of all hybrids were positive when stained for the presence of Thy-1.1 and Thy-1.2.

Nucleic acid techniques. Southern analyses were carried out by standard techniques (16). DNA was digested with the appropriate enzyme, electrophoresed in a 0.75% agarose gel, blotted onto nitrocellulose, and hybridized to a ^{32}P-labeled cDNA composed of the 5′ end of the Lyt-2 gene [*PstI-EcoRI* fragment of pcLy2-7b, kindly provided by Dr. J. Parnes (17)]. For dot blots, RNA was isolated by the guanidine hydrochloride method (18), transferred onto nitrocellulose, and probed as described above.

RESULTS

Lyt-2 expression is suppressed in hybridomas made from BW 5147 and Lyt-2⁺ SJL lymph node cells. Normal Lyt-2⁺ T cells were isolated from SJL lymph nodes and fused to BW5147, which expresses neither L3T4 nor Lyt-2 (Fig. 2, *panels 1* and *2*). It was thought that these fusions might result in induction of the silent BW5147 Lyt-2 gene or suppression of the expressed normal T cell gene. As a control, we examined expression of Thy-1, a T cell surface molecule expressed by both normal T cells and BW5147. Since there are two allelic forms of Lyt-2 in mice, Lyt-2.1, and Lyt-2.2, and also Thy-1, Thy-1.1, and Thy-1.2, it was possible for us to distinguish surface expression of the BW5147 genes from the normal T cell Lyt-2 and Thy-1 genes. Individual hybridomas were analyzed for surface expression of Thy-1.1, Lyt-2.1 (BW5147 alleles), Thy-1.2, Lyt-2.2 (SJL alleles), and L3T4. The results are shown in Figure 1. All of the hybridomas tested expressed both Thy-1.1 and Thy-1.2, confirming that they were, in fact, hybrids between BW5147 and SJL LNC (a representative hybrid is shown in Fig. 2, *panel 6*). We never observed the induction of the BW5147 Lyt-2.1 allele in these hybridomas, either alone or in conjunction with the L3T4 molecule, and none of the hybrids expressed the SJL Lyt-2.2 molecule alone. However, we did observe the coexpression of Lyt-2.2 and L3T4 on 16% of the hybridomas tested. It should be noted that the level of Lyt-2.2 and L3T4 expression in these hybrids was very low and that this low level expression was often transient (e.g., Fig. 2, *panel 4*). A small percentage (11% of the hybridomas expressed L3T4 alone (e.g., Fig. 2, *panel 5*). This result was not unexpected in light of the fact that the SJL LNC used for fusion were contaminated with 2 to 8% L3T4⁺ cells and previous fusions with normal L3T4⁺ T cells yielded hybridomas that always expressed the L3T4 molecule (data not shown). BW5147 is therefore "permissive" for the expression of L3T4. The surprising result was that the vast majority of hybridomas expressed neither Lyt-2 nor L3T4, despite the fact that the LNC used for fusion ranged from 89 to 98% Lyt-2.2⁺ cells (Fig. 2, *panel 3*). It appeared that expression of Lyt-2.2 from the normal SJL parent was lost upon fusion of Lyt-2⁺ SJL LNC to BW5147.

Lack of Lyt-2 expression is not due to loss of the gene encoding Lyt-2. It was important to show that lack of Lyt-2 expression in these Lyt-2⁻ hybridomas was not due to preferential loss of the incoming Lyt-2 gene. In order to do this it was necessary to distinguish the BW5147 from the SJL Lyt-2 gene. To this end, we identified a *HaeIII* restriction enzyme fragment length polymorphism, shown in Figure 3. DNA from SJL liver, AKR liver, or BW5147 was digested with *HaeIII*, separated in

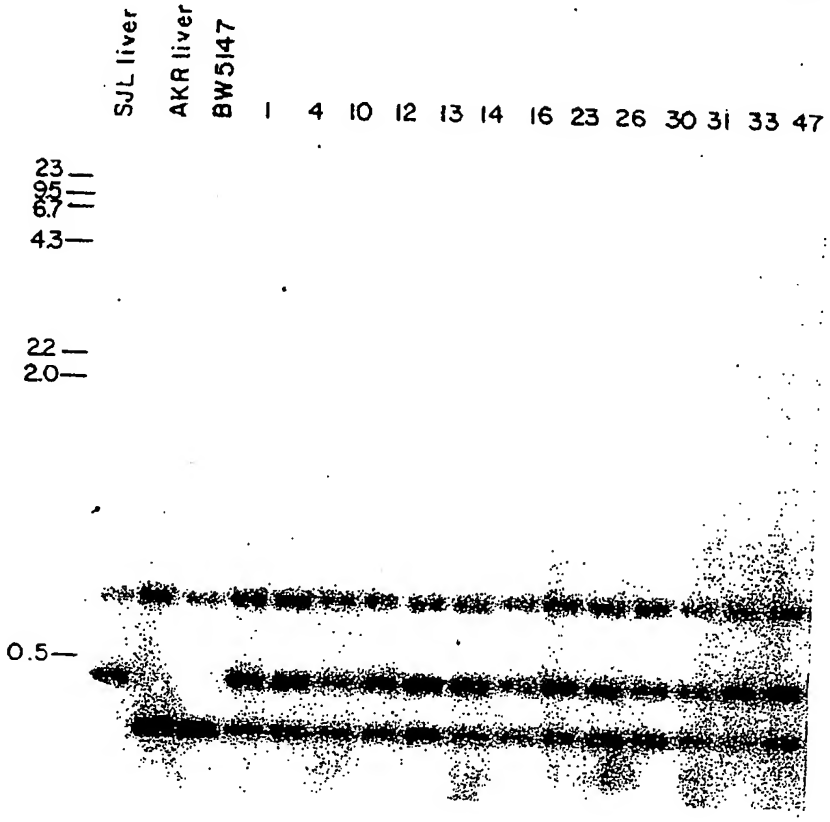


Figure 3. Lack of Lyt-2 expression is not due to loss of the gene encoding Lyt-2. DNA from parental sources (SJL liver, AKR liver, BW5147) was digested with *Hae*III and compared to DNA from 13 Lyt-2$^-$L3T4$^-$ hybridomas generated by fusion of Lyt-2$^+$ SJL LNC to BW5147.

an agarose gel, blotted onto nitrocellulose, and probed with a ^{32}P-labeled murine Lyt-2 cDNA. This analysis yielded a 0.4-kb band unique to SJL and a 0.35-kb band unique to BW5147. When DNA from 13 of the Lyt-2$^-$ hybridomas was analyzed in the same way, both the 0.4- and the 0.35-kb bands were present, indicating that the hybridomas retain at least one allele of the Lyt-2 gene from both the BW5147 and the SJL parent. Therefore, lack of Lyt-2 expression cannot be explained by loss of the gene encoding this cell surface molecule. Rather, it appears that the loss of Lyt-2 expression in Lyt-2$^-$ hybridomas is due to negative regulation by means of a *trans*-acting factor originating from the BW5147 genome.

Regulation of Lyt-2 expression is at the level of transcription. We next determined whether control of Lyt-2 expression in Lyt-2$^-$ hybridomas was at the level of transcription or translation. This was done by dotting total cellular RNA from several Lyt-2$^-$ hybridomas onto nitrocellulose and probing with ^{32}P-labeled Lyt-2 cDNA. RNA from five L3T4$^+$/Lyt-2$^-$ control hybridomas produced by fusion of BW5147 to class II-restricted, L3T4$^+$ T cells (AODH3.4, 3DO54.8, 3DT52.5, DO11.10, and SKK45.10) and from BW5147 showed no hybridization with the Lyt-2 cDNA probe (Fig. 4). RNA from two Lyt-2$^+$ murine tumor lines (VL3/1 and AKR1.G.1) hybridized strongly with the Lyt-2 probe and the intensity of hybridization correlated well with the level of surface Lyt-2 expression in these two tumors. In contrast, 11 surface Lyt-2$^-$ hybridomas expressed no detectable Lyt-2 transcripts by this analysis. These results suggest that the *trans*-acting factor controlling Lyt-2 expression in Lyt-2$^-$ hybridomas exerts its control at the transcriptional level, although we can not rule out the possibility that changes in Lyt-2 mRNA stability also contribute to the loss of detectable transcripts.

Loss of Lyt-2 expression is associated with the remethylation of DNA at sites 5′ of the Lyt-2 gene. In general, transcriptionally silent genes are heavily methylated, whereas transcriptionally active genes are undermethylated (19). We examined the methylation state of the Lyt-2 gene and 5′ sequences in order to determine whether the negative control exerted by BW5147 affected methylation of the Lyt-2 gene in Lyt-2$^-$ hybridomas. Most methylation in mammalian DNA occurs at the 5 position of cytosine in the dinucleotide CG (20). The isoschizomer pair of restriction enzymes, *Msp*I and *Hpa*II, is commonly used to study methylation in mammalian DNA. MspI cleaves the recognition sequence CCGG whether or not the DNA is methylated, whereas *Hpa*II cuts unmethylated DNA only.

To study methylation of the Lyt-2 gene and 5′ sequences, DNA from Lyt-2$^+$ (AKR1.G.1, Lyt-2$^+$LNC) and Lyt-2$^-$ (BW5147, AKR liver) cells was digested with *Bam*HI alone or in combination with either *Msp*I or *Hpa*II (Fig. 5*A*). *Bam* HI digestion resulted in an 8-kb fragment which encompasses the first 3 exons of the Lyt-2 gene (Dr. J. Parnes, personal communication) and approximately 6 kb of 5′ flanking sequences (Fig. 6). In all cases the 8-kb *Bam*HI fragment was reduced to 2.8 kb when

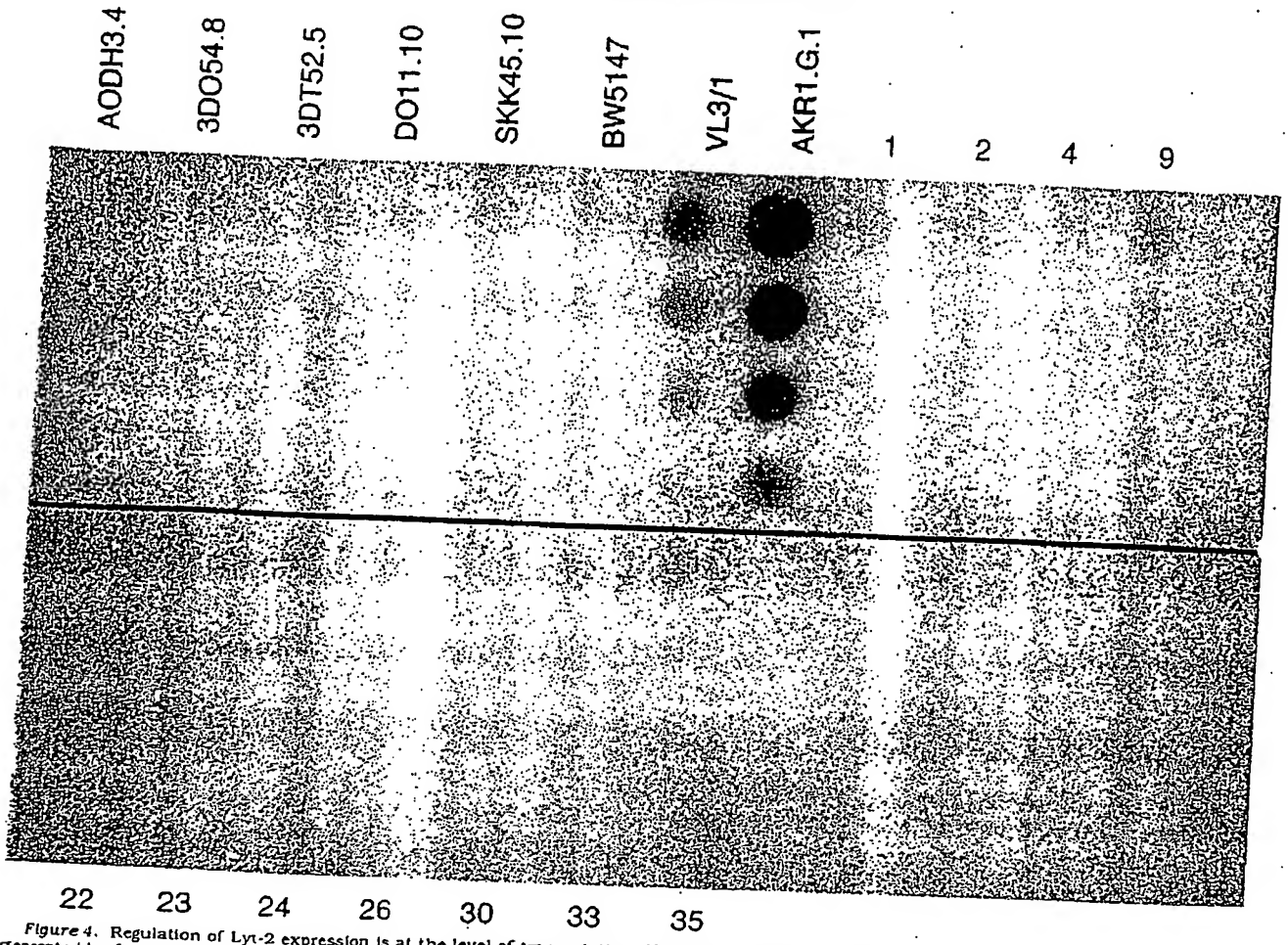
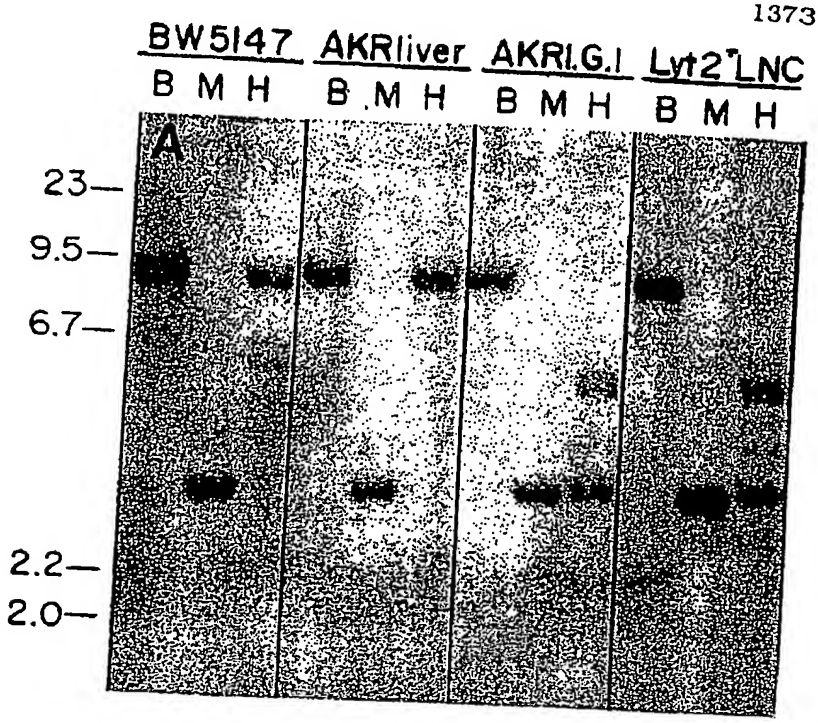


Figure 4. Regulation of Lyt-2 expression is at the level of transcription. Eleven Lyt-2$^-$L3T4$^-$ hybridomas (1, 2, 4, 9, 22, 23, 24, 26, 30, 33, 35), generated by fusion of Lyt-2$^+$ SJL LNC to BW5147, were tested for the presence of Lyt-2$^+$ message by dot blot analysis. Negative controls included RNA from several Lyt-2$^-$L3T4$^-$ hybridomas (AODH3.4, 3DO54.8, 3DT52.5, DO11.10, SKK45.10) as well as the parent thymoma, BW5147. Positive controls included RNA from VL3/1, an Lyt-2$^+$ lymphoma line (kindly provided by Dr. I. Weissman), and RNA from AKR1.G.1.Oua^R-1, an Lyt-2$^+$ thymoma (kindly provided by Dr. R. Hyman).

digested with *Msp*I. This band is the result of cleavage at the *Msp*I/*Hpa*II site located in exon 2 of the Lyt-2 gene and cleavage at an *Msp*I/*Hpa*II site located 5′ of the Lyt-2 gene (Fig. 6, *site 4*). The 8-kb band from AKR liver was not reduced when further digested with HpaII, demonstrating that CCGG sites within this region are methylated in the DNA of liver cells (Fig. 5*A*). The 8 kb band from BW5147 DNA remained largely intact following digestion with HpaII, although a faint 5.0-kb band, which represents a small amount of demethylation of the BW5147 Lyt-2 gene at *site 1*, was observed. In contrast, the 8-kb *Bam*HI fragment from Lyt-2 expressors (AKR1.G.1 and Lyt-2$^+$ LNC) was reduced upon digestion with *Hpa*II to two major bands of approximately 4.3 and 2.8 kb. We know from other experiments (data not shown) that the DNA of these Lyt-2 expressors is unmethylated at the internal *Msp*I/*Hpa*II sites. Therefore, the 2.8-kb band arises from cleavage at *Msp*I/*Hpa*II *site 4* and the 4.3-kb band arises from cleavage of a second *Msp*I/*Hpa*II site 5′ of the Lyt-2 gene (Fig. 6, *site 1*). These two sites, as well as the *Msp*I/*Hpa*II sites within the Lyt-2 gene, are undermethylated in Lyt-2$^-$ cells and methylated in Lyt-2$^-$ cells.

We next examined the methylation state of the Lyt-2 gene in Lyt-2$^-$ hybridomas. These hybridomas were made by fusing BW5147 to SJL Lyt-2$^+$ LNC; therefore, if the DNA were unchanged in terms of methylation, we would expect to see a superimposition of the BW5147 methylation pattern upon the Lyt-2$^+$ LNC pattern (i.e., bands of 8, 4.3, and 2.8 kb). Any deviation from this would indicate a change in the methylation state of the Lyt-2 gene in hybridoma DNA. Digestion of hybridoma DNA with *Bam*HI alone and with *Bam*HI followed by *Msp*I resulted in the expected 8-kb fragment (*Bam*HI) and 2.8-kb fragment (*Bam*HI + *Msp*I) (Fig. 5*B*, *lanes B and M*). Digestion with *Bam*HI followed by *Hpa*II resulted in various patterns (Fig. 5*B*, *lanes H*). The 8-kb fragment found in all hybrids presumably represents the uncut *Bam*HI fragment from the methylated BW5147 genome. The 4.3-kb fragment results from *Hpa*II digestion of the SJL *Bam*HI fragment. However, the expected 2.8-kb fragment found in DNA from SJL Lyt-2$^+$ LNC was completely lacking or greatly reduced in all Lyt-2$^-$ hybridoma DNA. Loss of this 2.8-kb fragment appears to result from the following



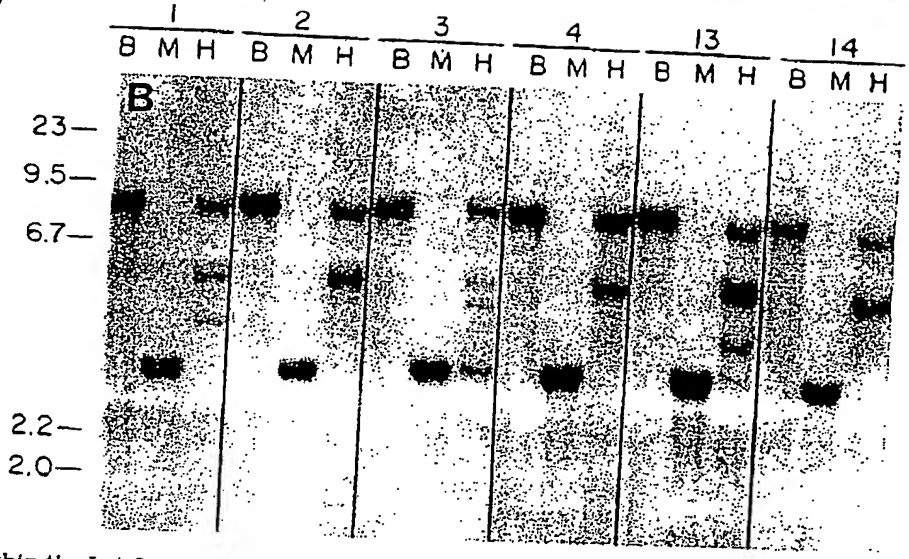


Figure 5. Loss of Lyt-2 expression is associated with the remethylation of DNA at two sites 5′ of the Lyt-2 gene. *A*, DNA from Lyt-2⁻ cells (BW5147, AKR liver) and Lyt-2⁺ cells (AKR1.G.1, Lyt-2⁺ LNC) was digested with *Bam*HI alone (*B*) or *Bam*HI followed by *Msp*I (*M*) or *Hpa*II (*H*). *B*, DNA from 6 Lyt-2⁻L3T4⁻ hybridomas (1, 2, 3, 4, 13, 14) was digested as described in (*A*). *C*, DNA from Lyt-2⁻ cells (BW5147, AKR liver, hybridomas 1,4,10) and Lyt-2⁺ cells (AKR1.G.1, Lyt-2⁺ LNC) was digested with *Bam*HI alone (*B*) or *Bam*HI followed by *Hha*I (*H*).

events (Fig. 6): 1) remethylation within the Lyt-2 gene to yield a 3.4-kb band (e.g., hybrids 1 and 13); 2) remethylation at *site 4* but not within the Lyt-2 gene to yield the 4.3-kb band (e.g., hybrids 2,4, and 14); 3) remethylation both within the Lyt-2 gene and at *site 4* to give a 5.0-kb band (e.g., hybrids 9, 21, and 31, data not shown); and 4) remethylation at *site 4* and cutting at a third *Msp*I/*Hpa*II site 5′ of the Lyt-2 gene (Fig. 6, *site 3*) to yield a 3.8-kb band (hybrid 3). (We do not yet know whether the 3′ end of the 3.8-kb fragment is the 3′ *Bam*HI site or whether it is at the *Msp*I/*Hpa*II cluster located within the Lyt-2 gene. In normal LNC site 3 is clearly methylated in the alleles of Lyt-2⁺ LNC DNA which yield the 4.3-kb fragment upon digestion with *Bam*HI followed by *Hpa*II, but this site could be either methylated or unmethylated in those alleles that result in the 2.8-kb fragment.) From these results we can conclude that *site 4*, as well as sites within the Lyt-2 gene, have been completely or partially remethylated in hybrid DNA derived from the Lyt-2⁺ normal T cell parent.

We have confirmed and extended this result by using *Hha*I, a methylation sensitive enzyme that cleaves GCGC but not G^{m}CGC. *Bam*HI-digested DNA from liver and BW5147 was not further cleaved by this enzyme, whereas DNA from AKR1.G.1 and Lyt-2⁺ LNC was reduced to a 4.4-kb band, indicating that a *Hha*I site within

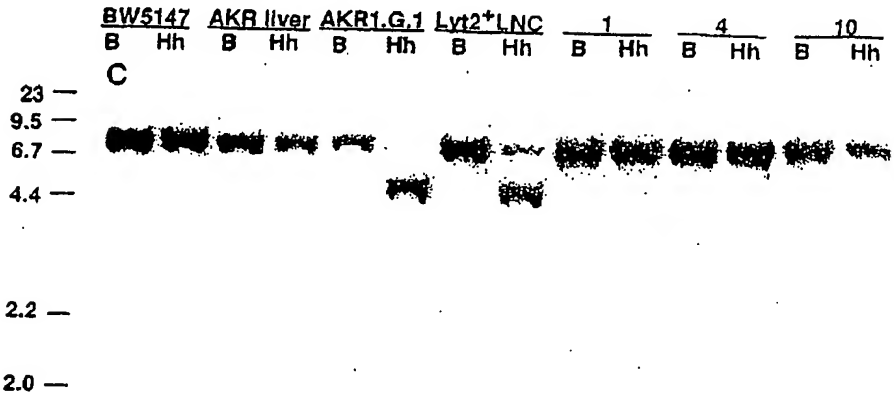


Figure 5C.

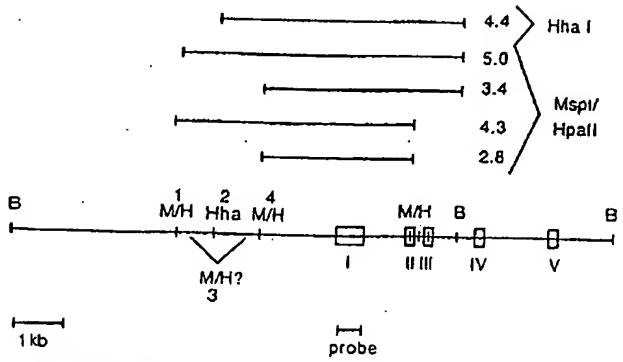


Figure 6. Restriction enzyme map of Lyt-2 gene and 5′-flanking region. *BamHI* sites (*B*), *MspI/HpaII* sites (*M/H*), *HhaI* site (*H*), exons encoding Lyt-2 (I–V) (33).

the 8-kb *BamHI* fragment is methylated in Lyt-2$^-$ cells and unmethylated in Lyt-2$^+$ cells (Fig. 5C). This *HhaI* site was remethylated in DNA from Lyt-2$^-$ hybridomas, as shown by the failure of this enzyme to cleave the 8-kb *BamHI* fragment. Thus, another site has been identified, located approximately 2.6 kb from the start of the Lyt-2 gene (Fig. 6, *site 2*), which is remethylated in Lyt-2$^-$ hybridomas.

DISCUSSION

In this paper we have shown that fusion of Lyt-2$^+$ T cells to BW5147 yielded hybrids that failed to express surface Lyt-2 or Lyt-2 mRNA, suggesting negative control by means of a *trans*-acting regulatory factor formed in BW5147. Negative control resulting from fusion to BW5147 appears to be specific for Lyt-2; expression of other T cell-specific molecules (e.g., L3T4, LFA-1, T-200, Thy-1, TAP, CD3, various lymphokines, and the T cell antigen receptor α, β, γ, δ genes) has been shown to be unaffected by fusion to BW5147. "Extinction," or loss of expression of tissue-specific traits after fusion of dissimilar cell types, is a well-established phenomenon (21, 22). Our observations extend these previous findings, since they concern regulation of gene expression after fusion of similar cell types (T cells and thymomas). In fact, the suppression of Lyt-2 expression upon fusion of Lyt-2$^+$ T cells to BW5147 may reflect processes that occur during normal T cell development, since expression of Lyt-2 is developmentally regulated. Early T cell precursors, the prothymocytes, express neither L3T4 nor Lyt-2: mature thymocytes and peripheral T cells express one or the other of these genes. Immature thymocytes, which may represent the intermediate stages between these two cell types, express both L3T4 and Lyt-2 (2–4). If BW5147 is derived from a prothymocyte or, more likely, since it contains functionally rearranged α and β TCR genes, if BW5147 is derived from an abnormal mature cell committed to L3T4 expression, then inhibition of Lyt-2 expression after fusion of Lyt-2$^+$ cells to the tumor may reflect processes going on either in prothymocytes or in mature T cells. Further work should indicate whether or not this idea is true.

Loss of methyl groups from the 5′ regions of mammalian genes in association with the expression of tissue-specific genes is a well-documented phenomenon (19). Several examples have been cited where de novo methylation of introduced viral and globin genes was associated with negative regulation of the incoming genes (23–30). In the experiments described here, negative regulation of an endogenous, T cell specific gene (Lyt-2) was accompanied by remethylation within, and at sites 5′ to, the Lyt-2 gene itself. A role for DNA methylation in regulation of other T cell surface molecules has recently been proposed. Richardson et al. (31) treated humar. T4$^-$(human L3T4 analog)T8$^+$(human Lyt-2 analog) T cells with 5-azacytidine, which inhibits methylation of newly synthesized DNA, and thereby induced expression of T4. Sneller and Gunter (32) proposed that methylation, caused by ethylmethanesulfonate, of sequences 5′ to the Thy-1 gene caused loss of Thy-1 expression by EL-4 T-cell lymphoma cells. We have yet to determine whether in our system remethylation is the cause of suppression of Lyt-2 transcription, whether it is part of a more complex sequence of regulatory events leading to inhibition of Lyt-2 transcription, or whether it is a consequence of changes in chromatin structure brought about by a regulatory mechanism yet to be defined. However, these experiments do provide a new system for the study of *trans*-acting factors affecting gene expression, and may also shed some light on events that occur during normal T cell development.

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DECLARATION BY DR. MURIEL MOSER

I, Muriel Moser, declare as follows:

I hold a Ph.D. degree in Zoology with greatest distinction from the Free University of Brussels, Belgium. Specialized in zoology since 1977, I am heading a research group focusing on the physiology of antigen presenting cells (since 1986) at the free University of Brussels. I hold, since April 2002, a degree of "Agrégé de l'enseignement supérieur" I frequently lecture at international meetings and I am a regular reviewer for several international Journals. I author over one independent, world-wide patent application and over 50 international peer-reviewed publications in the field of cell-based immunology. I have initiated the study of murine dendritic cells at the free university of Brussels and I am experimenting since the filing date of the present patent application onwards successfully with the production of human DC/tumor hybrids/hybridomas and their use to eliminate cancer in patients. I am the past president of the French group on dendritic cells. I was appointed several times as the European expert on Immunology. I enclose my Curriculum Vitae in annex (Enclosure 1).

I am one of the inventors of the US patent application US 09/802,397 and have reviewed and understand all prior art and Office Actions of record. The present Declaration illustrates that the present invention relies on unexpected results, therefore this declaration may be helpful for the examiner in advancing prosecution.

1. The claimed subject-matter

The present invention relates to a method of producing an anti-tumor response in a mammalian subject, said method comprising administering to said subject a plurality of dendritic cell/tumor hybrids and/or a dendritic cell/tumor hybridoma. The present invention illustrates for the first time that said cells may be produced and carry characteristics of tumor cells and DCs which makes them interesting for cancer therapy.

Furthermore, the present invention proves for the first time that tumors are efficiently eliminated using said hybrids/hybridomas. In particular, I have shown in this present application that said approach may easily be followed, efficient and applicable in humans.

Before the filing of the applications whereto the above-mentioned patent application claims priority (US 09/049,502; 09/025,405; 08/625,507 and 08/414,480) nobody gave the experimental/clear proof for the generation of dendritic cells (DC)/tumor hybrids or hybridomas. In particular, nobody described hybrids/hybridomas which may be applied in animal, especially human, therapy.

The present invention further teaches that DC/tumor hybrids/hybridomas may be produced efficiently starting from proliferating dendritic cells or a dendritic cell at a more immature stage. Until now, there is no cellular characteristic or marker available which may be used to define this preferred DC-fusion partner. The only definition which may apply is that said cells may be proliferating DC or are DCs at a more immature stage.

Furthermore, in order to make DC/tumor hybrids allowable in human medicine, it is essential that no essential body-part(s) of the patient is(are) used (such as spleen). A

solution to this problem is also formulated in the present invention. The present invention suggests to use cells found in for instance bone-marrow, lymph/lymph nodes or in blood. According to the present invention said cells may be proliferating dendritic cells or a dendritic cell at a more immature stage as mentioned above. Said dendritic cells may originate from induced DC-progenitors.

The approach to make human DC/tumor hybrids/hybridomas, which is applicable in human medicine, has never been suggested before the filing of any of the US applications to which the present US patent application claims priority to. Based on the prior art it was not predictable that by using dendritic cells, or, proliferating dendritic cells or a dendritic cell at a more immature stage, as described in the present application, hybrids/hybridomas could be made having both the DC and tumor characteristics which are needed to trigger tumor elimination in a patient.

2. Non-obviousness of the subject-matter of claims 1 and 3 over Guo et al. (1994) in view of Somasse et al. (1992)

I respectfully disagree with the assertion in the outstanding Office Action that that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the dendritic cells taught by Somasse et al. (1992) for the B cells of Guo et al. (1994) as the APC fusion partner in producing a dendritic cell/tumor cell hybrid, and, that it would be obvious to administer said product to a subject for production of an anti-tumor response.

In my opinion, based on said documents it is not predictable that such hybrids could be made and which characteristics said hybrids would carry. Furthermore, I am convinced that the approach of Guo for making his hybrids may not be followed to produce hybrids which may be used for human applications. As the method of making said hybrids (and thus also the starting cells) are different, it is clear that the resulting hybrids will be different. These different aspects are discussed in the paragraphs below.

The difference between the present invention and the teachings of Guo and Somasse lays thus in the definition of the hybrids AND in their use.

a/ The feasibility of making DC/tumor hybrids is not predictable

Changing the fusion partner of the tumor cell, as with the B cell of Guo et al. (1994), to another antigen-presenting cell does not allow one of skill in the art to predict the outcome of such an experiment. As mentioned in the discussion section of Carbone et al. (1988, see copy in annex (Enclosure 2), p 1374, first column, second paragraph, l.10-12), extinction or loss of expression of tissue specific traits after fusion of dissimilar cells is a well-established phenomenon (Lewin 1980; Killary and Fournier 1984). This negative regulation is not specific for cell fusions but was also observed for incoming genes (Clough et al. 1982; Palmiter et al. 1982; Gautch and Wilson 1983; Groffen et al. 1983; Hardies et al. 1983; Manor 1985; Humphries et al. 1985; Dyson et al. 1985) (p 1374, second column, second paragraph, l.1-7 of Carbone, et al.). Methylation has been shown to be a commonly used system to regulate expression within the cell; it has even been shown to be used for the natural regulation of certain T-cell surface molecules (Richardson et al. 1986) (p 1374, first column, second paragraph, l.10-15 of Carbone, et al.).

Only after extensive experiments carried out by my group it was possible to conclude that fusing tumor cells with dendritic cells as described in the present application can result in the generation of hybrids/hybridomas that carry tumor antigenic markers combined with various dendritic specific markers. The success of this fusion could not have been predicted by the prior art, and in fact, there would be sufficient reasons for one of ordinary skill in the art to believe that such a fusion of two dissimilar cells would not work, based on the above-described phenomenon of loss of expression of tissue specific traits after fusion. One of ordinary skill in the art would have taken a very cautious attitude and would not have predicted a successful outcome for this experiment until it had been demonstrated.

In this respect, it should be recognized by the Examiner that experimental evidence for the possibility to produce an anti-tumor response in a subject comprising administering dendritic cell/tumor cell hybrids/hybridomas was given for the first time in the parent of the present U.S. patent application. To reason that the outcome of the present experiments would have been predictable in advance or would be obvious is only possible when one reasons with hindsight. It is clear from the reasons cited above that the skilled person would not have reasoned in this way and would not have predicted any outcome of such an experiment before it had been proven that it works.

I hereby respectfully submit that it was not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the dendritic cells taught by Somasse et al. (1992) for the B cells of Guo et al. (1994) as the APC fusion partner in producing a dendritic cell/tumor cell hybrid. Therefore I respectfully submit that the administration of the hybrids/hybridomas of the present invention to a patient in order to produce an anti-tumor response in said patient is not obvious over Guo et al. (1994) in view of Somasse et al. (1992).

b/ The approach of Guo cannot be followed to produce hybrids/hybridomas for in vivo treatments of animals (humans)

Further, in support of the inventive step of especially claims 1 and 3, I wish to point towards the impossibility to use the method used in Guo et al. (1994) for the production of hybrid cells for animal (especially human) applications.

The method according to Guo et al. (1994) involves the use of B cells as fusion partners of the tumor cells. Said B-cells were recovered from the spleen of rats earlier injected with with soluble antigen in complete Freund's adjuvant, which cannot be applied in humans. In addition, if immunizations are done without Freund's adjuvant, the outcome of the B cells remains unpredictable in individual animals and it is expected to be unpredictable in individual human patients.

Furthermore, the approach followed by Guo does not allow multiple booster applications. In this respect I wish to stress the difference between the method used to generate hybridomas and the method used in Guo et al. (1994). In the present invention hybridomas are selected by growing them in selection medium. Unfused immortalized cell lines are killed by the exposure to a drug. In the description of the present application the use of the HAT (hypoxanthine-aminopterin-thymidine) selection medium is illustrated. After selection, the hybridoma can be cultured when needed and used for multiple booster vaccinations. This is a major advantage compared to the strategy used by Guo et al. (1994) where fusions are made and immediately used for treatment without making them immortalized;

each treatment needs a separate fusion step where variability in cell population between booster applications can be generated.

A further advantage of the DC/tumor hybrids/hybridomas of the present invention over the hybridomas of Guo et al. is that no essential body parts are needed as start material, that the fusion partners are easy to isolate, that it allows the treatment of a subject using cells which are compatible with its immune system and that the approach of the present invention allows to produce a product for which a guarantee of its composition and quality may be given. All these aspects are discussed in detail in section 3 (see below).

3. Non-obviousness of the subject-matter of claims 19-26 over Guo et al. (1994) in view of Sornasse et al. (1992)

I am of the opinion that the note of the Examining Division in respect of the fact that the spleen cells of Guo et al. (1994) and Sornasse et al. (1992) would also comprise an isolated DC as well as the only two known murine subtypes of DC (i.e. myeloid and lymphoid, both of which are derived from bone marrow) is inappropriate.

I would like to point out that both myeloid and lymphoid DC may be considered as isolated DCs.

Claims 19-20 and 47-50

It is true that all DCs found *in vivo* (thus also from spleen) are originally bone marrow derived. However, what the present application teaches is different. The present application illustrates that DC-cells derived from bone marrow, lymph/lymph nodes, blood or other tissues are a better alternative to spleen cells to start the production of the hybrids of the present invention.

I confirm hereby that my group has the experience that a DC-preparation from spleen is not a good start population to aim for the production of DC/tumor hybrids/hybridomas. Indeed I have the experience that primary cultured DCs (proliferating DCs) are preferentially needed (e.g. cultured from bone marrow cells) to produce said hybridomas; using primary DCs (non-proliferating DCs; e.g.isolated from spleen) will not result in the efficient and reproducible production of DC/tumor hybrids/hybridomas. I have the experience that when using preparations of spleen cells mainly hybrids may be formed between non-DCs (for example T cells) and tumor cells even if the DC population in said preparation is dominant. Consequently, I am of the opinion that starting from mouse splenic cells is extremely doubtful that a real DC/tumor hybridoma can be obtained.

In addition, bone narrow, lymph/lymph nodes and blood may contain DC-precursors or intermediates between monocytes and DCs. Said monocytes may be used to further differentiate into proliferating intermediates between monocytes and DCs or into proliferating DCs before the production of the hybrids/hybridomas of the present invention. These intermediates or precursor cells are not present in tissues such as spleen.

In the section below I further explain that the method applied by Guo to produce the DC/tumor hybridomas may not be applied in medicine (humans or animals) and thus has no industrial value.

A further optimization of DC/tumor hybrids of the present invention, is that said hybrids/hybridomas may be applied in medicine (human and animal). Indeed, the DC used to make the hybrids/hybridomas of the present invention are purified from cells without the need for isolating essential organs/cells from said mammals such as spleen. Using spleen cells, as used in the method of Guo et al, 1994, inherently results in the killing of the organism from which said cells are retrieved. Contrarily, the use of DC fusion partners derived from blood, lymph/lymph nodes or bone marrow as proposed in the present invention allows to keep the organism, from which said cells are isolated, alive.

Furthermore, a patient may be treated with cells derived fom his own body making the cancer treatment compatible with his immune system. A higher efficiency of the therapy is therefore also expected.

In summary, the use of DC-fusion partners purified from blood, lymph/lymph node or bone marrow cells allows a more efficient production of the hybrids/hybridomas of the present invention. In addition, these bone marrow and blood cells are easy to isolate and allow a better approach for human applications. I also refer to the arguments given in section 2/b (above).

Claims 21-28

I hereby explain the cellular origin of existing DCs in animals.

DC precursor cells from bone marrow may be considered as the stem cell from which DC myeloid precursor cells (e.g. monocytes) and DC lymphoid precursor cells may differentiate. Both precursor cells may give rise to differentiated, also called mature DCs; myeloid and lymphoid DC respectively.

Mature myeloid and lymphoid DCs may be found in specific tissues, such as spleen, but may also be present in for instance blood. Precursor cells of myeloid or lymphoid origin may be present all over the body. However, it is accepted in the scientific literature that these are not present in specific tissues, such as spleen.

Most stem cells (bone marrow) or precursor cells are present in a resting state. This means that these are non-proliferating. However, proliferation may be induced (in vivo or in vitro) in said cells.

I have the experience that the hybrids/hybridomas of the present invention may be made with high efficiency when differentiating blood, lymph/lymph nodes or bone marrow DC-precursors or proliferating DCs (before they enter in the resting state) are used. Said cells are therefore considered as preferable fusion partner for the production of the hybrids/hybridomas of the present invention (new claims 29-46).

4. Non-obviousness of the subject-matter of claims 5-10 over Guo et al. (1994) in view of Sornasse et al. (1992) and in further view of U.S. Patent 5,851,756.

As explained above, skilled person may not derive from Guo et al. and Sornasse that such a DC/tumor hybrid may be formed.

In addition, I respectfully disagree that based on the '756 patent one of ordinary skill in the art at the time of the invention would have been motivated to induce DC-characteristics (using GM-CSF) in DC/tumor hybrids/hybridomas before using said fused cells in anti-tumor treatments.

This is non-obvious based on the following reasons:

1/ The induction in the method of the present invention is performed *after* the hybrid formation, the DCs corresponding to the DCs of the '756 patent do not exist anymore.

2/ The DCs of the '756 patent are different from the hybrids of the present invention. It is not obvious that such a hybrid would behave like the isolated DC cell of the '756 patent.

3/ The induction allows to induce the expression of DC characteristics, not to increase the number of DC cells in blood as taught in the '756 patent and explicitly repeated by the Examining Division.

5. Non-obviousness of the subject-matter of claims 11-14 over Guo et al. (1994) in view of Somasse et al. (1992) and in further view of U.S. Patent 5,637,483.

I respectfully disagree that based on the '483 patent an ordinary skilled in the art at the time of the invention would have been motivated to irradiate the hybrids/hybridomas of the present invention to prevent proliferation.

According to the '483 patent, the irradiation of the tumor vaccine is presented as an essential step. I hereby refer to the claims of said patent and to especially example 6 of said patent. In said example, vaccination studies using life transduced tumor cells are discussed. In said experiment (column 14, l.48-50) it is explicitly mentioned that all tumor cells, except the IL-2 secreting tumor cells, resulted in tumor formation. It is obvious that life-tumor-cells would never be accepted as vaccine in human therapy.

Contrarily, in the present invention it is suggested that said hybrids/hybridomas may be irradiated. However, said irradiation is not an essential step in the production of said vaccine. Indeed, the hybrids/hybridomas of the present invention are fusions between tumor cells and DC cells. They may have predominant tumor characteristics, predominant DC characteristics, or an equal distribution of both characteristics. For cells with especially predominant DC characteristics, said irradiation may have a more negative effect on the anti-cancer therapy. The present invention teaches explicitly that also living hybrids/hybridomas elicited an anti-tumor immune response (paragraph [0100], l.9-12 of the application as published). Therefore, said cells have predominantly DC characteristics and do not need the irradiation step as proposed in the '483 patent before it is used in the anti-cancer therapy.

Said irradiation should be considered as an essential step in the approach of the '483 patent, and is optional in the method of the present invention. This proves that the hybrids of the present invention are clearly different from the tumor cells of the '483 patent, and that the characteristics of said hybrids are not predictable based on the tumor cells of the '483 patent.

I am convinced that the teachings of the present invention and the '756 patent are not comparable.

Declaration Dr. Moser
US Patent Application No. 09/802,397

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true.

Signed this 7 day of November, 2003

Muriel Moser

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